Characterization of Two Cyclic Metabolites of Sitagliptin

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ABSTRACT:

Two novel metabolites of the dipeptidyl peptidase inhibitor sitagliptin (MK-0431, (2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine), were identified after purification from dog urine. The metabolites (referred to as M2 and M5) were characterized by hydrogen/deuterium exchange tandem mass spectrometry and NMR spectroscopy nuclear Overhauser effect experiments as the cis and trans stereoisomers formed by cyclization of the primary amino group with the alpha carbon of the piperazine ring, following oxidative desaturation.

Sitagliptin (Januvia), also known as MK-0431, (2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine, was a selective, potent DPP-4 inhibitor (Kim et al., 2005), recently approved for the treatment of type 2 diabetes. Sitagliptin has been shown to inhibit plasma DPP-4 activity in normal volunteers (Bergman et al., 2005, 2006; Herman et al., 2005b, 2005a) and patients with type 2 diabetes (Herman et al., 2005b; Scott et al., 2006). The pharmacokinetics, metabolism, and excretion of sitagliptin in rats, dogs, and humans are described elsewhere (Beconi et al., 2006; Vincent et al., 2007), and in patients with type 2 diabetes (Herman et al., 2005a), and to significantly reduce HbA1c, and fasting plasma glucose in patients with type 2 diabetes (Herman et al., 2005b; Scott et al., 2006). The pharmacokinetics, metabolism, and excretion of sitagliptin in rats, dogs, and humans are described elsewhere (Beconi et al., 2007; Bergman et al., 2006; Vincent et al., 2007). The objective of the present study was to characterize two metabolites of sitagliptin, referred to as M2 and M5. As discussed by Beconi et al. (2007) and Vincent et al. (2007), M2 and M5 were minor metabolites in vitro (rat, dog, monkey, and human liver microsomes and hepatocytes) and in vivo, where they were detected at very low levels in rat and human plasma, and rat, dog, and human excreta. However, M2 and M5 were relatively abundant in dog plasma, especially at the later time points, comprising 4 to 56% of circulating radioactivity between 1 and 24 h after oral administration of [14C]sitagliptin. M2 and M5 were, therefore, purified for further characterization, including evaluation of their pharmacological activity.

Materials and Methods

Chemicals. Sitagliptin (Fig. 1), (2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine, was prepared by Process Research at Merck Research Laboratories (Rahway, NJ). [14C]Sitagliptin was synthesized by the Labeled Chemistry Group at Merck Research Laboratories. The radiochemical purity was 98.8%, as determined by HPLC, with a specific activity of 132 Ci/mg. For dosing of dogs, [14C]sitagliptin was diluted with unlabeled solvent used was deuterated methanol.

Assay of DPP-4 Activity. The purified M2 and M5 were tested for DPP-4 inhibition using a published procedure (Leiting et al., 2003). In brief, small quantities of the purified M2 and M5 were dissolved in D_2O and then infused into a Thermo Electron LCQ Deca XP ion trap mass spectrometer. The LCQ was operated in the electrospray positive ion mode. The source voltage was maintained at 5 kV. Data acquisition and reduction were carried out using Xcalibur software (version 1.2; Thermo Electron).

NMR Analysis. Proton and nuclear Overhauser effect spectroscopy NMR spectral analyses of M2 and M5 were carried out at room temperature on a Varian Inova 600 MHz NMR spectrometer (Varian, Inc., Palo Alto, CA). The solvent used was deuterated methanol.

DPP-4 Activity. The purified M2 and M5 were tested for DPP-4 inhibition using a published procedure (Leiting et al., 2003). In brief, the metabolites were tested for inhibition of hydrolysis of 50 μM Gly-Pro-7-amino-4-methylcoumarin (substrate) by 50 pM DPP-4 at 37°C for 30 min in a 100 mM HEPES buffer (pH 7.5) in the presence of 0.1 mg/ml bovine serum albumin.

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Results and Discussion

Mass Spectra of Sitagliptin and Metabolites M2 and M5. Sitagliptin exhibited a protonated molecule at \( m/z \) 408 \([M + H]^+\) in electrospray positive ionization mode. Upon collision-induced dissociation (CID), it afforded three major fragments at \( m/z \) 235, 193, and 174, which were assigned as indicated in Fig. 2. The product ion at \( m/z \) 193, assigned to a fragment derived from the triazolopiperazine moiety, was useful in the determination of modifications to this portion of the molecule. A minor fragment was observed at \( m/z \) 391, attributed to the loss of \( \text{NH}_3 \). Metabolites M2 and M5 exhibited the same protonated molecule with \( m/z \) 406, which was 2 mass units lower than that of the parent compound, and identical CID spectra. The most intense fragment ion was detected at \( m/z \) 191; this fragment was 2 mass units lower than the corresponding fragment of the parent compound. Also, there was no evidence of loss of \( \text{NH}_3 \). Three possible structures can be proposed based on the liquid chromatography-MS/MS data. M2 and M5 could have been formed either through desaturation of the carbon-carbon bond of the piperazine ring or by desaturation of the C–N bonds of the piperazine ring followed by cyclization via the primary amine. C–N desaturation can occur with either one of the two carbons at the alpha position to the amide nitrogen. The change in the fragmentation pattern of M2 and M5, including the absence of \([M + H - \text{NH}_3]^+\) ion \((m/z \) 389\), in comparison to the parent compound supports the latter.

Characterization of M2 and M5 by H/D Exchange MS/MS and NMR. Due to the limited information obtained from the mass
The H/D exchange MS/MS experiment was conducted as described previously (Liu et al., 2001). Small quantities of the purified M2 and M5 were dissolved in D2O and infused into an ion trap mass spectrometer for analysis using an electrospray probe. If M2 and M5 were formed through desaturation of the carbon-carbon bond of the piperazine ring, the neutral molecules would contain two exchangeable hydrogens from the primary amino group. On the other hand, if the primary amine had been converted to a secondary amine via C–N desaturation followed by cyclization, only one exchangeable hydrogen would be present in the neutral molecules. The experiments proved that the latter was true for both M2 and M5. The protonated molecules [M + H]+ in H2O were observed at m/z 406, whereas their deuterated ions in D2O were observed at m/z 408 (Fig. 3). This confirmed the cyclic structures for M2 and M5. A similar conclusion was derived from the H/D exchange MS/MS data on the fragment at m/z 191, which became m/z 192 in D2O, indicating that it did not contain any exchangeable hydrogens (aside from the proton charge) since it contained a C-N double bond (Fig. 3). On the other hand, one would expect to see m/z 193 (one exchangeable hydrogen) if it contained a carbon-carbon double bond.

Proton NMR spectral data of sitagliptin, M2, and M5 are summarized in Table 1. In the proton NMR spectrum of sitagliptin, the methylene f protons of the triazolopiperazine ring were observed at 4.94 and 5.02 ppm. These signals were missing from the spectra of M2 and M5, which contained, instead, a methane signal at 5.74 and 5.83 ppm, respectively. This finding indicated that one of the protons of the original methylene f in the parent compound had been lost. In the NMR spectrum of M2, this isolated methine proton Hf showed nuclear Overhauser effect with the g proton at 3.23 ppm, as well as the methine proton Hg, where the amine group is attached. These data allowed the unambiguous assignment of the structure for M2 shown in Table 1, where Hf and Hg are in the cis configuration. M5 exhibited a proton NMR spectrum similar to that of M2, with slight differences in the chemical shifts of some of the protons (Table 1). M5 lacked nuclear Overhauser effect between Hf and Hg, which suggested that M5 is the trans isomer of M2, i.e., Hf and Hg protons were in the trans configuration.

**DPP-4 Inhibition by M2 and M5.** The purified M2 and M5 showed less than 50% inhibition of DPP-4 activity at the concentration tested (20 μM), indicating that their IC50 values were greater than 20 μM. Given that the parent sitagliptin has an IC50 of 18 nM (Kim et al., 2005), M2 and M5 are at least 1000-fold less potent than sitagliptin.

### TABLE 1

Assignments of 1H NMR chemical shifts (ppm in CD3OD) of sitagliptin and M2 and M5 (integral, multiplicity, coupling constants in Hz)

<table>
<thead>
<tr>
<th>Protons</th>
<th>Sitagliptin</th>
<th>M2</th>
<th>M5</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>7.19 (1H, m)</td>
<td>7.13 (1H, m)</td>
<td>7.09 (1H, m)</td>
</tr>
<tr>
<td>b</td>
<td>7.38 (1H, m)</td>
<td>7.36 (1H, m)</td>
<td>7.37 (1H, m)</td>
</tr>
<tr>
<td>c</td>
<td>3.08 (2H, m)</td>
<td>2.87 (1H, dd, 14.1, 7.3)</td>
<td>2.91 (1H, dd, 14.4, 7.0)</td>
</tr>
<tr>
<td>d</td>
<td>3.85 (1H, m)</td>
<td>3.47 (1H, m)</td>
<td>3.21 (1H, m)</td>
</tr>
<tr>
<td>e</td>
<td>2.92 (2H, d, 6.2)</td>
<td>2.44 (1H, dd, 17.2, 3.8)</td>
<td>2.29 (1H, dd, 16.7, 3.7)</td>
</tr>
<tr>
<td>f</td>
<td>4.94 (1H, d, 17.5)</td>
<td>2.21 (1H, dd, 17.2, 11.7)</td>
<td>2.21 (1H, dd, 16.7, 10.6)</td>
</tr>
<tr>
<td>g</td>
<td>5.02 (1H, d, 17.5)</td>
<td>5.74 (1H, s)</td>
<td>5.83 (1H, s)</td>
</tr>
<tr>
<td>h</td>
<td>5.02 (1H, d, 17.5)</td>
<td>5.74 (1H, s)</td>
<td>5.83 (1H, s)</td>
</tr>
</tbody>
</table>

* Signal splitting patterns: s = singlet, d = doublet, t = triplet, dd = double doublet, td = triple doublet, add = double double doublet, and m = multiplet.

* Signals become complex because of the existence of two forms of sitagliptin arising from restricted rotation of the amide bond.
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References


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